Congenic Mesenchymal Stem Cell Therapy Reverses Hyperglycemia in Experimental Type 1 Diabetes

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OBJECTIVE—A number of clinical trials are underway to test whether mesenchymal stem cells (MSCs) are effective in treating various diseases, including type 1 diabetes. Although this cell therapy holds great promise, the optimal source of MSCs has yet to be determined with respect to major histocompatibility complex matching. Here, we examine this question by testing the ability of congenic MSCs, obtained from the NOR mouse strain, to reverse recent-onset type 1 diabetes in NOD mice, as well as determine the immunomodulatory effects of NOR MSCs in vivo.

RESEARCH DESIGN AND METHODS—NOR MSCs were evaluated with regard to their in vitro immunomodulatory function in the context of autoreactive T-cell proliferation and dendritic cell (DC) generation. The in vivo effect of NOR MSC therapy on reversal of recent-onset hyperglycemia and on immunogenic cell subsets in NOD mice was also examined.

RESULTS—NOR MSCs were shown to suppress diabetogenic T-cell proliferation via PD-L1 and to suppress generation of myeloid/inflammatory DCs predominantly through an IL-6-dependent mechanism. NOR MSC treatment of experimental type 1 diabetes resulted in long-term reversal of hyperglycemia, and therapy was shown to alter diabetogenic cytokine profile, to diminish T-cell effector frequency in the pancreatic lymph nodes, to alter antigen-presenting cell frequencies, and to augment the frequency of the plasmacytoid subset of DCs.

CONCLUSIONS—These studies demonstrate the inimitable benefit of congenic MSC therapy in reversing experimental type 1 diabetes. These data should benefit future clinical trials using MSCs as treatment for type 1 diabetes. ***Diabetes*** **59:3139–3147, 2010**

Mesenchymal stem cell (MSC) therapy has in recent years emerged as a promising treatment modality for diseases with immune etiology, particularly given the increasing appreciation for the morbidity associated with immunosuppression. MSCs have been demonstrated to exhibit profound immunomodulatory effects in vitro and in vivo, and these immunomodulatory capabilities have been shown to be exerted through both direct contact and production of soluble markers (1–4). Moreover, upregulation of B7-H1/PD-L1 by IFN-γ has been shown to play a central role in the immunosuppressive properties of MSCs via direct contact with activated T-cells (5,6). In vitro studies have also demonstrated the ability of MSCs to regulate the function of T-cell effector pathways through promotion of regulatory dendritic cell (DC) generation, due to MSC-modulated alteration of DC cytokine profiles as evidenced by increased production of regulatory cytokines such as IL-10 and reduction of inflammatory cytokines including IFN-γ, IL-12, and TNF-α, thereby inducing a more anti-inflammatory or tolerant DC phenotype (7,8). These immunomodulatory effects as well as an extensive capacity for in vitro expansion of MSCs have prompted the launch of numerous clinical trials (1). MSC therapy has yielded promising results in the treatment of graft versus host disease (GVHD) as well as in the resolution of Crohn's disease-associated fistulas, in stabilization of refractory progressive multiple sclerosis, and in reversal of multiorgan dysfunction in patients with systemic lupus erythematosus (9–12). However, although the therapeutic value of MSCs for attenuating the autoimmune disorder type 1 diabetes has logical potential, MSC treatment of this particular disease remains largely unexplored. Trials using MSC therapy in patients with type 1 diabetes are underway, yet these efforts have been initiated in the near absence of preclinical data. In this regard, we and others have recently demonstrated delayed onset of experimental type 1 diabetes as well as reversal of recent-onset diabetes in response to allogeneic MSC therapy, whereas in our study administration of autologous diabetic MSCs showed no beneficial effect (13,14). Our previous work also indicated that congenic NOR MSCs imparted the greatest benefit in preventing type 1 diabetes. The NOR/LtJ strain, while resistant to insulitis due to the protective Idd alleles (15), shares the diabetogenic $H2^{g7}$ complex with the NOD/LtJ strain. NOR mice are 85% homologous to spontaneously diabetic NOD mice and are thus somewhat analogous to nondiabetic siblings of type 1 diabetic patients. Here, we sought to further examine the therapeutic efficacy of NOR MSCs on reversal of recent-onset diabetes and to elucidate the mechanisms by which NOR MSCs may act to ameliorate diabetes pathogenesis.

RESEARCH DESIGN AND METHODS

Mice. NOR/LtJ, NOD/LtJ, and NOD.Cg-Tg(TcraBDC2.5)1DoiTg(TcrbBDC2.5)2Doi/DoiJ (BDC2.5) were purchased from the Jackson Laboratories (Bar Harbor, ME). All procedures used in animal experiments were in accordance with the standards set forth in the Guidelines for the Care and Use of Laboratory Animals at Harvard University.

MSC culture and differentiation. To generate NOR MSCs, bone marrow mononuclear cells were isolated from the femurs and tibiae of NOR/LtJ mice. Cells were seeded in tissue culture flasks at a concentration of $1 \times 10^6/cm^2$ as previously described (13) and were trypsinized at 80% confluence and consolidated 2:1 until passage 4 (P4); from P4 to P6, cells were used for injection, characterization, or in vitro assays. 7.5×10^5 MSCs/well were

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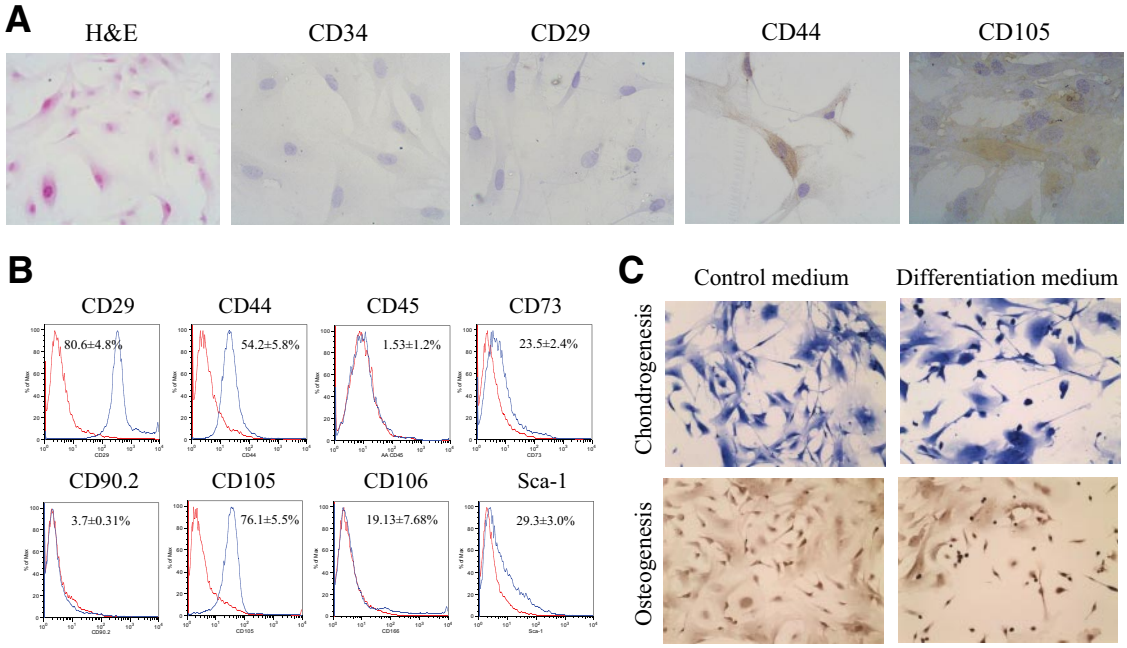

FIG. 1. Characterization of NOR MSCs. *A*: Immunohistochemical staining of NOR MSC cultures demonstrates fibroblast cell morphology by hematoxylin–eosin staining, substantial expression of the MSC markers CD44 and CD105, moderate expression of CD29, and lack of expression for the hematopoietic stem cell marker CD34. *B*: Flow cytometric analysis of NOR MSC P4 cultures (n = 5, data shown as mean ± SEM) shows abundant expression of the classical MSC markers CD29, CD44, and CD105, while MSCs were negative for the hematopoietic lineage-restricted markers CD45 and CD90.2. Sca-1, CD73, and VCAM (CD106) were expressed at moderate levels. *C*: NOR MSCs were shown to undergo osteogenesis and chondrogenesis after exposure to differentiation factors. (A high-quality digital representation of this figure is available in the online issue.)

cultured for 48 h in 6-well plates with 0.05, 0.5, or 5 ng/ml recombinant murine IFN-γ (Peprotech, Rocky Hill, NJ). MSC differentiation to mesodermal tissues was performed as previously described (13).

Flow cytometric analysis. MSCs were analyzed for surface expression of a battery of markers at P4. Anti-mouse antibodies purchased from BD Biosciences (San Jose, CA) included CD45, Ly-6A/E/Sca-1, CD44, CD90.2, and CD73. Antibodies purchased from eBioscience (San Diego, CA) were CD105, CD29, CD106/VCAM-1, PD-1, B7-H1/PD-L1, and B7-H2/PD-L2. For ex vivo studies, the spleen and pancreatic lymph nodes (PLNs) were harvested and subjected to analysis for CD4 effectors, CD8 effectors, and Tregs as previously described (13). Splenocytes or DC cultures were also stained with CD11c, CD11b, F4/80, CD45R/B220, CD40, CD80, CD86, and Ly-6c (BD Biosciences). The biotinylated lineage panel was purchased from Miltenyi Biotec (Auburn, CA), and cells were secondarily stained with streptavidin (BD Biosciences).

Immunohistochemistry. Adherent NOR MSCs were fixed on slides and stained with hematoxylin–eosin for morphological evaluation, as previously described (13). Immunohistochemistry was also performed as previously described (13).

T-cell receptor-stimulated proliferation. NOD $CD4^+$ T-cells were isolated by magnetic bead separation using CD4 microbeads (Miltenyi Biotec). 1×10^5 $CD4^+$ T-cells were stimulated with 1 μg/ml anti-mouse CD3 and anti-mouse CD28 (BD Biosciences) alone or in combination with 1×10^4, 2×10^4, or 4×10^4 control or IFN-γ-challenged (0.05, 0.5, or 5 ng/ml for 48 h) NOR MSCs or NOR splenocytes in 96-well plates for 48 h, followed by pulsing with 1 μCi tritiated thymidine (Perkin Elmer, Waltham, MA) for 16 h. Tritium uptake was assessed using a MicroBeta FilterMate-96 Harvester and a 1,450 MicroBeta TriLux (both from Perkin Elmer).

Autoreactive T-cell proliferation. BDC2.5 $CD4^+$ cells were extracted from isolated splenocytes using magnetic bead separation (Miltenyi Biotec, Auburn, CA), and the BDC2.5 autoreactive T-cell assay was performed as previously described (13). In experiments with siRNA knockdown, PD-L1 or nontargeting pool siRNA were added to MSCs using the Accell platform (Dharmacon, Lafayette, CO) for 3 days prior to culturing with BDC2.5 T-cells, NOD DCs, and BDC2.5 islet peptide, followed by pulsing with 1 μCi tritiated thymidine as above.

ELISPOT. The autoreactive T-cell assay was performed as above using NOR MSCs in an ELISPOT assay as previously described (16). ELISPOT kits (BD Biosciences) to assess IFN-γ and IL-6 production were used according to the manufacturer's instructions.

Luminex and ELISA. To assess cytokine production of murine serum and culture supernatant samples, a 21-plex cytokine kit (Millipore, St. Charles, MO) was used according to the manufacturer's instructions and as previously described (16). To assess production of IFN-α, M-CSF, and Flt3L, murine ELISA kits (R&D Systems, Minneapolis, MN) were used according to the manufacturer's instructions.

DC culture. Bone marrow-derived NOD DCs were generated as previously described (16). In DC and MSC coculture, 1×10^5 NOR MSCs were plated at day 0 of NOD DC culture. For blocking studies, 10 μg/ml anti-IL-6 (eBioscience) was added at day 0 and replaced on days 3 and 6 at the same time as media additions or changes.

Giemsa staining. Cytospins of DC cultures at day 8 were stained with Giemsa stain (Sigma-Aldrich, St. Louis, MO) according to the manufacturer's instructions, and images were obtained using a Nikon TE300 system.

Reversal studies. Female NOD mice were monitored beginning at 10 weeks of age, and on day 2 of hyperglycemia (>240 mg/dl), a sustained-release insulin pellet (LinBit, LinShin Canada, Inc., Ontario, Canada) was placed subcutaneously into the dorsum. The initial MSC injection (1×10^6 cells i.v.) was injected within 24 h of pellet placement, and 1×10^6 NOR MSCs were injected intravenously twice per week thereafter for 4 weeks. Normoglycemia was maintained as needed during the last two weeks of treatment by 250 ng/dl insulin (Lantus, Sanofi-Aventis, Bridgewater, NJ). Mice were monitored daily by measuring blood glucose until the time of sacrifice, and measurements were performed by tail bleeding according to National Institutes of Health guidelines.

RESULTS

NOR MSC phenotype is consistent with mesenchymal lineage. NOR MSCs were evaluated by immunohistochemical and flow cytometric analysis for their expression of classical MSC markers as well as costimulatory molecules. Cultured cells were shown to be positive for the MSC markers CD29, CD105, and CD44 but were negative for the hematopoietic lineage-restricted marker CD34 after immunohistochemical staining (Fig. 1*A*). Surface staining revealed substantial expression of CD29, CD44, and CD105, with moderate expression of CD73 and Sca-1, and MSCs were found to be negative for the leukocyte antigen CD45 (Fig. 1*B*). Our NOR MSCs were additionally found to be capable of differentiating into cells of mesodermal

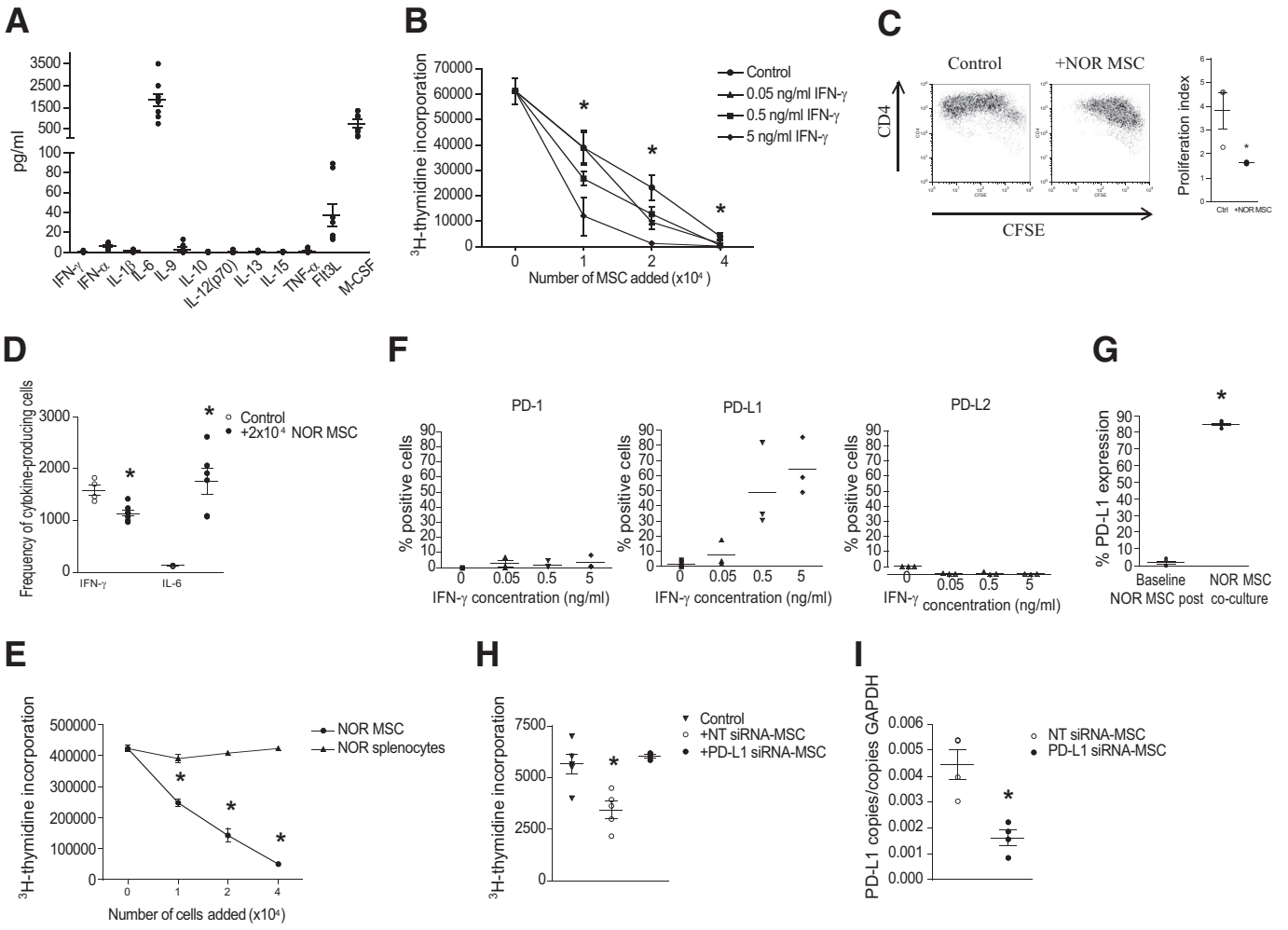

FIG. 2. NOR MSC suppression of diabetogenic autoreactive T-cells via PD-L1. *A*: Cytokine studies of NOR MSC cultures revealed considerable levels of IL-6, with M-CSF and Flt3L produced at lesser but substantial levels in comparison to other growth factors ($n = 4$). *B*: NOR MSCs suppressed TCR-stimulated proliferation of NOD CD4$^+$ cells in a dose-dependent manner, in which increasing numbers of IFN-γ-stimulated NOR MSCs were added to 1×10^5 NOD CD4$^+$ cells in the presence of 1 μg/ml anti-CD3 and anti-CD28 ($n = 5$, $P < 0.027$ for 1×10^4 MSCs, $P < 0.0001$ for 2×10^4 and 4×10^4 MSCs), and IFN-γ challenge enhanced the suppressive effect of NOR MSCs. (*C*) 2×10^4 NOR MSCs were shown to significantly reduce autoreactive T-cell proliferation ($n = 5$, $P = 0.047$), as evaluated by CFSE dilution and calculation of proliferation index when added to a BDC2.5 autoreactive assay containing BDC2.5 CD4$^+$ T-cells, NOD DCs, and 100 ng/ml BDC2.5 islet peptide. *D*: IFN-γ production was similarly suppressed in the presence of 2×10^4 NOR MSCs by ELISPOT in the BDC2.5 autoreactive assay ($n = 5$, $P = 0.0024$), while IL-6 levels were enhanced ($P = 0.0005$). *E*: Addition of 1, 2, or 4×10^4 NOR splenocytes had no suppressive effect on anti-CD3/-CD28 T-cell proliferation as compared with addition of identical numbers of NOR MSCs ($n = 4$, $P < 0.0001$ for NOR MSCs, not significant for NOR splenocytes). *F*: NOR MSCs stimulated with 0.05, 0.5, or 5 ng/ml recombinant murine IFN-γ show dose-dependent upregulation of PD-L1 expression by flow cytometric analysis ($n = 3$, $P < 0.008$ for 0.5 and 5 ng/ml), a minor increase in PD-1 (p = not significant), and no increase in expression of PD-L2. *G*: NOR MSCs after coculture with BDC2.5 CD4$^+$ T-cells, NOD DCs, and BDC2.5 peptide exhibited marked upregulation of PD-L1 expression at 72 h by flow cytometric analysis ($n = 5$, $P < 0.0001$). *H*: siRNA knockdown of PD-L1 in MSCs abrogated the suppressive effect observed on autoreactive T-cell proliferation when 2×10^4 MSCs were added ($n = 6$, $P = 0.0034$ for control versus nontargeting siRNA, not significant for control versus PD-L1 siRNA for representative experiment shown), and (*I*) gene expression analysis of MSCs demonstrated efficient suppression of PD-L1 transcripts in response to siRNA treatment ($n = 4$, $P = 0.016$). Experiments were performed between 3 and 6 times, and data are displayed with means and SEM.

lineage (Fig. 1*C*). These data confirm that our cultured NOR MSCs are phenotypically and lineally mesenchymal as well as functionally multipotent.

NOR MSCs suppress autoantigen-specific and nonspecific T-cell proliferation. To further characterize NOR MSCs, we performed cytokine studies of NOR MSC cultures, which demonstrated the presence of various cytokines, with IL-6 most notable in its production ($n = 4$, Fig. 2*A*). As MSCs are defined by their immunomodulatory ability as well as their surface marker profile and multipotent potential, we added increasing numbers of NOR MSCs previously challenged with increasing concentrations of IFN-γ to an anti-CD3-/anti-CD28-stimulated proliferative assay using NOD CD4$^+$ T-cells to assess the capacity of NOR MSCs to suppress T-cell proliferation. NOR MSCs potently inhibited T-cell receptor (TCR)-stimulated proliferation in a dose-dependent manner (Fig. 2*B*, $n = 5$, $P < 0.027$ for indicated conditions), and pretreatment with IFN-γ enhanced suppression of proliferation dose-dependently. To examine whether NOR MSCs are able to specifically suppress autoreactive T-cells, MSCs were added to an autoreactive T-cell assay, in which isolated BDC2.5 CD4$^+$ T-cells, or H2^{g7}-restricted diabetogenic T-cells, are cocultured with NOD DCs and BDC2.5 islet peptide. As shown in Fig. 2*C*, NOR MSCs significantly suppressed autoreactive T-cell proliferation, as assessed by carboxyfluorescein succinimidyl ester (CFSE) dilution and calculation of proliferation index ($n = 5$, $P = 0.047$). Addition of

NOR MSCs was also able to suppress production of IFN-γ by BDC2.5 T-cells, which has been described previously as a characteristic of proinflammatory autoreactive T-cells (17), and the production of IL-6 was shown to be enhanced in the presence of NOR MSCs (Fig. 2*D*, $n = 6$, $P = 0.0024$ for IFN-γ, $P = 0.0005$ for IL-6). These data demonstrate that not only are NOR MSCs capable of suppressing T-cell proliferation stimulated through the TCR via anti-CD3/-CD28 stimulation but that, in the specific context of diabetogenic T-cells, NOR MSCs have a potent immunomodulatory effect on autoreactive T-cell proliferation and production of IFN-γ. NOR splenocytes were also tested for their suppressive ability in all of the assays above to assess the effects of congenicity, and no effects on anti-CD3/-CD28-stimulated T-cell proliferation, autoreactive T-cell proliferation, or autoreactive T-cell IFN-γ production were observed (Fig. 2*E* [$n = 4$, $P < 0.0001$ for NOR MSCs, not significant for NOR splenocytes], and data not shown, respectively).

Suppression of autoreactive T-cell proliferation by NOR MSCs is mediated by PD-L1. Recent studies have highlighted the central role of the negative costimulatory PD-1 pathway ligand PD-L1 in suppressing the proliferation of autoreactive T-cells and in consequently halting the progression of type 1 diabetes in NOD mice (18–21). Given these data, we hypothesized that inhibition by PD-L1 serves as a mechanism by which NOR MSCs exert their immunomodulatory effects on diabetogenic T-cells. We thus examined the expression of PD-L1 on resting NOR MSCs and after activation with IFN-γ to evaluate expression of components of the PD-1 pathway in the context of inflammation. Although NOR MSCs expressed PD-L1 at insubstantial levels at baseline, stimulation with increasing doses of recombinant murine IFN-γ resulted in prodigious upregulation of PD-L1 expression in a dose-dependent manner, while PD-1 and PD-L2 expression underwent modest changes after IFN-γ challenge (Fig. 2*F*, $n = 3$, $P = 0.0002$ for PD-L1 expression of baseline compared with 5 ng/ml IFN-γ). We also investigated whether addition of unstimulated NOR MSCs to the BDC2.5 autoreactive assay would result in upregulation of PD-L1, as substantial IFN-γ was detected in the autoreactive T-cell experiments (Fig. 2*D*); indeed, coculture of NOR MSCs with autoreactive T-cell assay components resulted in considerable NOR MSC expression of PD-L1 (Fig. 2*G*, $n = 5$, $P < 0.0001$), indicating that an autoreactive inflammatory milieu induces marked upregulation of PD-L1 on MSCs. To determine the functionality of PD-L1 expression in the suppression of autoreactive T-cell proliferation by NOR MSCs, we treated NOR MSCs with PD-L1 siRNA prior to adding MSCs to our autoreactive T-cell assay. Indeed, treatment of NOR MSCs with PD-L1 siRNA abrogated the suppressive effect of MSCs on BDC2.5 T-cell proliferation (Fig. 2*H*, $n = 6$; $P = 0.0034$ for control versus nontargeting siRNA, not significant for control versus PD-L1 siRNA for representative experiment shown). Addition of greater numbers of PD-L1 siRNA-treated NOR MSCs (4 and 8×10^4; $n = 4$) to the autoreactive T-cell assay did not result in restoration of the suppressive effect of NOR MSCs (data not shown), perhaps due to the fact that MSCs have been shown to exert their immunomodulatory effects through cell contact. To confirm that our siRNA treatment resulted in efficient knockdown of PD-L1 expression, siRNA-treated MSCs were examined for PD-L1 copy number by real-time PCR. As shown in Fig. 2*G*, PD-L1 transcripts were significantly decreased by PD-L1 siRNA treatment of MSCs ($n = 4$, $P = 0.016$), demonstrating effective suppression of PD-L1. These data indicate that PD-L1 plays a significant role in the specific context of MSC-mediated suppression of diabetogenic T-cells.

NOR MSCs inhibit in vitro DC differentiation via IL-6. Aberrant DC development and imbalance in antigen-presenting cell (APC) subsets have been reported to be responsible for the lack of tolerance mechanisms in NOD mice (22,23). To examine the effect of NOR MSCs on DC generation, we performed in vitro studies of NOR MSCs and NOD DCs using an established method of DC culture (16,24). Because of the substantial production of IL-6 in our NOR MSC cultures (Fig. 2*A*) as well as the fact that IL-6 has been demonstrated to both suppress and alter DC differentiation (2,7,25,26), we performed IL-6 blocking studies in conjunction with coculture of MSCs and DCs. The presence of MSCs strikingly reduced CD11c and CD11b expression of DCs, so that the predominant population induced by MSCs was $CD11c^{low}CD11b^{low}$ (Fig. 3*A*, $CD11c^+CD11b^+$ cells = 40.7 ± 2.6% and 22.1 ± 2.4% for control and NOR MSC-treated, respectively, $n = 5$, $P = 0.0007$). Addition of anti-IL-6 somewhat abrogated the change in phenotype observed in DC coculture with NOR MSCs (Fig. 3*A*, $n = 4$, not significant in comparison to control [−/−] and in comparison to MSCs alone [−/+]). Of note, costimulatory molecule expression in the $CD11c^+$ population was not found to be significantly different in the presence or absence of MSCs (data not shown). The $CD11b^+$ population was also evaluated with respect to Ly-6c expression, as both $CD11b^+Ly\text{-}6c^{high}$ and $CD11b^+Ly\text{-}6c^{int}$ cells have been demonstrated to be inflammatory monocytes recruited to sites of inflammation (22,27). Coculture with NOR MSCs resulted in downregulation of both the $CD11b^+Ly\text{-}6c^{high}$ and $CD11b^+Ly\text{-}6c^{int}$ populations (Fig. 3*A*, $n = 4$, $P = 0.0053$ and $P = 0.02$, respectively), and this difference was again abrogated by blockade of IL-6. We then assessed the number of lineage-negative cells as a function of progenitor frequency or lack of differentiation, and NOR MSC treatment was shown to increase lineage-negative cells as well as increase the expression of Sca-1 within the lineage-negative population (Fig. 3*B*, $n = 4$, $P = 0.004$ and $P = 0.0085$, respectively). Treatment with anti-IL-6 was somewhat efficacious in abrogating the suppression of differentiation observed in response to MSC coculture, suggesting that other factors may be involved in the effect of MSCs on DC differentiation. We therefore examined the supernatants of DC and MSC cocultures at day 8 for cytokine production. As shown in Fig. 3*C*, coculture with MSCs significantly enhanced IL-6 levels ($n = 4$, $P = 0.0074$), and addition of IL-6 blocking antibody efficiently suppressed IL-6 production. Moreover, Flt3L and M-CSF production was increased in response to MSCs ($n = 4$, $P = 0.03$ and $P = 0.04$, respectively), and IL-6 blockade had no effect on increased levels of these cytokines ($P = 0.013$ and $P = 0.018$, respectively, in comparison to DCs alone, Fig. 3*C*). Conversely, production of TNF-α, a growth factor involved in the maturation of DCs as well as a cytokine secreted by mature DCs (16,28), was reduced in the presence of MSCs (Fig. 3*C*, $n = 4$, $P = 0.0056$), and blocking of IL-6 resulted in abrogation of this effect. To examine the morphology of DCs in response to MSCs, we performed Giemsa staining of day 8 DC cultures and

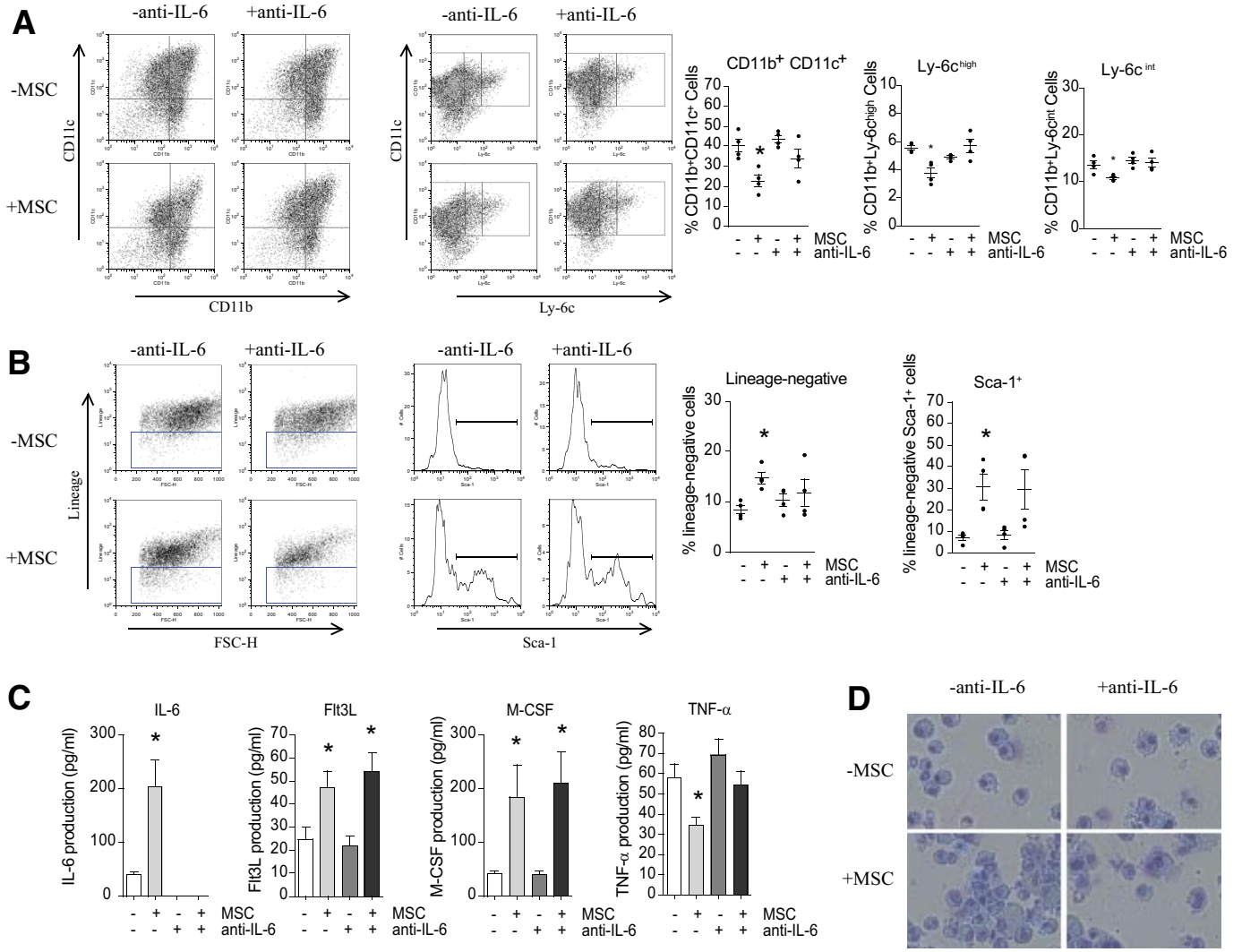

FIG. 3. MSC suppression of DC differentiation. *A*: Using an established model of DC generation from NOD bone marrow mononuclear cells, coculture with NOR MSCs was shown to markedly reduce the $CD11c^+CD11b^+$ population, so that the predominant cell phenotype was $CD11c^{low}CD11b^{low}$ ($CD11c^+CD11b^+$ cells = 40.7 ± 2.6% and 22.1 ± 2.4% for control and NOR MSC-treated, respectively, $n = 5$, $P = 0.0007$), whereas treatment with anti-IL-6 in large part abrogated this effect ($CD11c^+CD11b^+$ cells = 30.9 ± 4.7%, not significant in comparison to control [−/−] and MSCs alone [+/−]). Analysis of expression of Ly-6c in the $CD11b^+$ fraction demonstrated that coculture with NOR MSCs resulted in a decrease in both the $CD11b^+Ly\text{-}6c^{high}$ and $CD11b^+Ly\text{-}6c^{int}$ populations ($n = 4$, $P = 0.0053$ and $P = 0.02$, respectively), which was fully abrogated by blockade of IL-6 (p = not significant). *B*: The population of lineage-negative cells was evaluated in DC culture as a function of progenitor frequency; coculture with MSCs increased the percentage of Lin^- cells ($n = 4$, Lin^- cells = 8.94 ± 0.87% and 13.73 ± 1.08% for control and NOR MSC-treated, respectively, $P = 0.004$), which was in part rescued by addition of anti-IL-6 (p = not significant). Similarly, Sca-1 expression within the lineage-negative population was markedly increased in the presence of MSCs ($n = 4$, Lin^- $Sca\text{-}1^+$ cells = 6.98 ± 1.27% and 30.53 ± 6% for control and NOR MSC-treated, respectively, $P = 0.0085$). Although treatment with anti-IL-6 resulted in loss of significance of this effect, IL-6 blockade appeared to be incompletely effective in reducing Sca-1 expression in response to MSCs. *C*: Cytokine analysis of cocultures of DCs and NOR MSCs demonstrated marked IL-6 production in the presence of MSCs ($n = 4$, $P = 0.0074$) as well as efficient blockade of IL-6 in response to treatment with anti-IL-6. Both Flt3L and M-CSF levels were substantially increased in response to MSC coculture ($P = 0.03$ and $P = 0.04$, respectively), and IL-6 blockade had no effect on these growth factors ($P = 0.013$ and $P = 0.018$, respectively, in comparison to DCs alone). Conversely, TNF-α production was reduced in the presence of MSCs ($P = 0.0056$), and anti-IL-6 treatment resulted in abrogation of this effect (p = not significant). *D*: Giemsa staining of DC culture cytospins demonstrated a lower nuclear/cytoplasmic ratio in response to coculture with MSCs, and IL-6 blockade appeared to in large part abrogate this effect. Experiments were performed between 3 and 5 times, and data are displayed with means and SEM. (A high-quality color representation of this figure is available in the online issue.)

found that the nuclear-to-cytoplasmic ratio appeared to decrease after coculture with MSCs, a feature commonly associated with earlier stages of differentiation (29), and IL-6 blockade appeared to partially reverse this effect (Fig. 3*D*). Examination of side scatter of DCs by flow cytometric analysis revealed that coculture with MSCs resulted in a dramatic reduction in the degree of granularity (data not shown), again demonstrating a lack of differentiation in response to MSCs (30). Addition of MSCs to plasmacytoid DC (pDC) cultures resulted in enhanced pDC frequency, and this effect was fully reversed by IL-6 blockade (supplementary Fig. 1 in the online appendix available at http://diabetes.diabetesjournals.org/cgi/content/full/db10-0542/DC1), as IL-6 has been previously demonstrated to be important for pDC generation (31). Taken together, these data demonstrate a marked effect of NOR MSCs on DC phenotype, differentiation, and cytokine production, which is in large part mediated by IL-6.

NOR MSCs efficiently reverse recent-onset experimental autoimmune type 1 diabetes. Given our previous data in which congenic NOR MSCs were found to be most

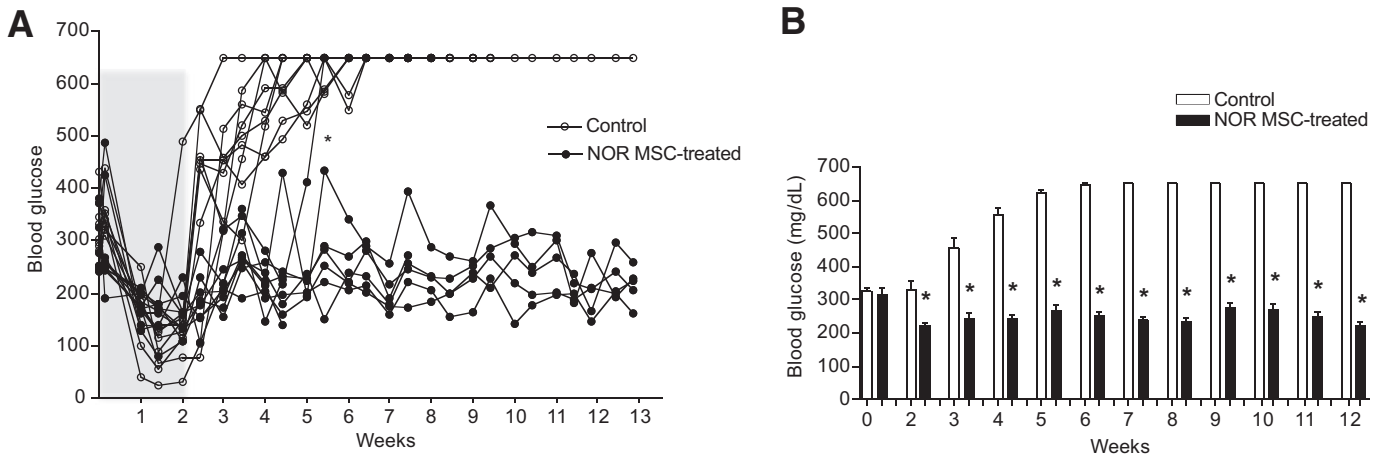

FIG. 4. NOR MSC therapy induces long-term reversal of recent-onset hyperglycemia. *A*: NOD mice were monitored beginning at 10 weeks of age, and on day 2 of hyperglycemia (blood glucose > 240 mg/dl), an insulin pellet was inserted subcutaneously for maintenance of normal glycemia during treatment. Mice were randomized to control or NOR MSC-treated groups; for NOR MSC treatment, 1×10^6 cells were injected intravenously twice per week for 4 weeks, and blood glucose measurements were taken daily. Eight of nine NOD mice treated with NOR MSCs exhibited reversal of diabetes. Five of six treated mice followed for 12 weeks maintained their reversal, whereas controls reverted to hyperglycemia (>600 mg/dl) soon after dissolution of the insulin pellet. *B*: Means of cumulative blood glucose measurements demonstrate no difference in level of hyperglycemia at days −1 and 0 (p = not significant), while weekly mean measurements beginning at week 2 after the initiation of treatment show significant decreases in blood glucose in response to NOR MSC therapy ($P < 0.001$ for all from week 2 to week 12). Data are displayed with means and SEM.

effective in preventing onset of diabetes in the NOD mouse model in comparison to autologous or allogeneic MSC treatment (13), we sought to determine the efficacy of NOR MSCs in reversing recent-onset hyperglycemia in NOD mice. Our previous work demonstrated that reversal of hyperglycemia in response to BALB/c MSC therapy, although effective, was only temporary, perhaps due to eventual rejection of the allogeneic cells (13). Using a treatment protocol identical to that of our previous study, we observed reversal of recent-onset hyperglycemia in eight of nine NOD mice treated with NOR MSCs at the 5-week point of post-treatment observation (Fig. 4*A*). Of note, only one mouse in the NOR MSC-treated group exhibited blood glucose levels > 600 mg/dl (* in Fig. 4*A*) at ~5 weeks into treatment. Whereas two NOD mice treated with NOR MSCs succumbed to unexplained deaths with no evidence of remission break, the other mouse was killed. Although several weeks of observation suffices for most reversal studies, the remaining five mice were observed for an extended period to ensure that their reversal was maintained (i.e., with no return to hyperglycemia). At 12 weeks after treatment, their average blood glucose measurement was 222 ± 13.4 mg/dl. All NOD mice (both NOR MSC-treated and controls) were provided a slow-release insulin pellet to allow for a limited period of metabolic recovery, yet in contrast to NOR MSC-treated mice, all control NOD mice ($n = 9$) redisplayed hyperglycemia almost immediately after dissolution of the insulin pellet. Weekly mean blood glucose measurements of treated mice were significantly reduced compared with controls from 2 to 12 weeks; notably, eight of nine hyperglycemic untreated mice died within 6 weeks after the onset of diabetes (Fig. 4*B*, $P < 0.001$ for all time points, one surviving control is shown beyond week 6.5). No treatment bias was present, as glucose measurements at the initiation of treatment did not differ between groups (Fig. 4*B*, P = not significant). These data demonstrate efficient and long-term reversal of recent-onset hyperglycemia in response to NOR MSC therapy.

NOR MSC treatment augments regulatory cytokine levels and induces regulatory DCs. To elucidate the mechanisms by which reversal of recent-onset hyperglycemia occurred in response to NOR MSC therapy, we performed serum cytokine studies after the completion of MSC administration at days 0, 7, 14, and 21. As shown in Fig. 5*A*, NOR MSC treatment resulted in increased circulating levels of IL-6, IL-7, IL-10, and IL-12(p40) (n = 3–5 samples, $P < 0.00065$ where indicated). Of note, IL-12(p70) levels were decreased in serum of NOR MSC-treated mice, but the difference did not reach statistical significance (data not shown). Flow cytometric analysis of the spleen and PLNs of treated and control mice at 2 weeks after the initiation of treatment demonstrated a reduction in the populations of $CD4^+CD44^{high}CD62L^{low}$ and $CD8^+CD44^{high}CD62L^{low}$ effector T-cells (Fig. 5*B*, $n = 5$, $P = 0.041$ and $P = 0.0022$ for CD4 and CD8 effectors, respectively), while no effect on Tregs was detected. In studies in which NOD mice were treated for 2 weeks with NOR MSCs and in which the proliferative capacity of isolated CD4 and CD8 T-cells in response to concanavalin A and anti-CD3/-CD28 stimulation was examined, no differential results were observed in proliferation of CD4 or CD8 T-cells isolated from splenocytes of NOR MSC-treated or control mice (data not shown). In light of our in vitro DC data, we analyzed the splenic APC populations in control and NOR MSC-treated mice. CD11c single-positive and $CD11c^{high}CD11b^+$ cells were found to be reduced in frequency in response to treatment with NOR MSCs (Fig. 5*C*, $n = 5$, $P = 0.004$ and $P = 0.015$, respectively). Fewer macrophages, identified as $F4/80^+CD11c^-$, were also found in NOR MSC-treated mice (Fig. 5*C*, $n = 5$, $P = 0.015$). Conversely, the $CD11c^{low}CD11b^+$ population was increased after NOR MSC therapy (Fig. 5*C*, $n = 5$, $P = 0.029$). The $CD11b^+$ population also showed reduced expression of Ly-6c after treatment with NOR MSCs (Fig. 5*C*, n = 3–5, $P = 0.042$). Further analysis of the CD11c single-positive DC population revealed a dramatic increase in B220 expression in this subset in response to NOR MSC treatment (Fig. 5*C*, $n = 5$, $P = 0.0094$), and pDCs of this phenotype have been shown to promote tolerance and to delay the onset of diabetes (32).

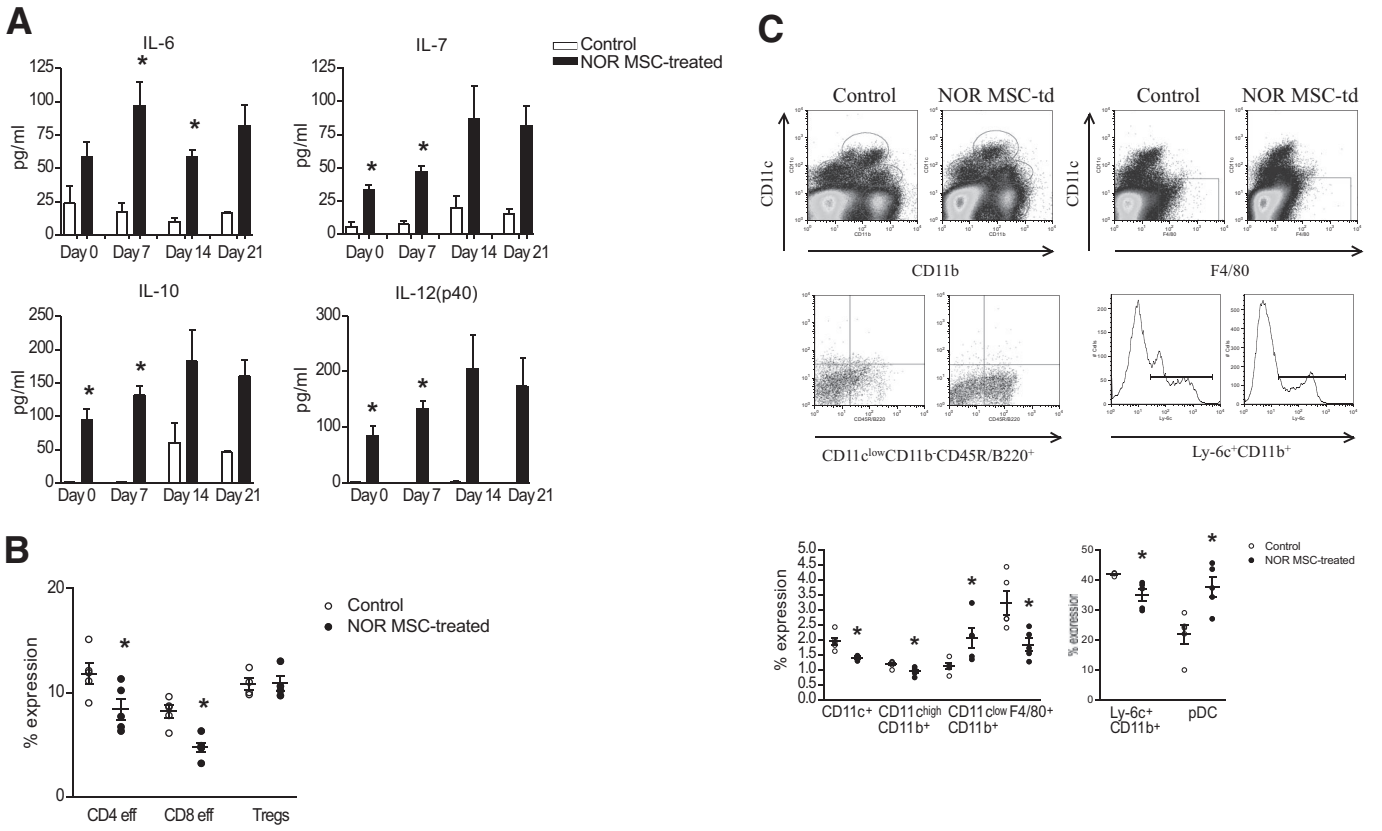

FIG. 5. NOR MSC therapy alters DC phenotype, cytokine profile, and effector cell frequency in vivo. *A*: Serum cytokine studies of NOR MSC-treated and control mice were performed at days 0, 7, 14, and 21 after completion of our treatment protocol at 4 weeks (n = 3–5 samples). NOR MSC therapy increased circulating levels of IL-6 at days 7 and 14 ($P < 0.0065$), increased IL-7 levels at days 0 and 7 ($P < 0.00021$), increased levels of IL-10 at days 0 and 7 ($P < 0.0052$), and increased levels of IL-12(p40) at days 0 and 7 ($P < 0.00091$). *B*: CD4 and CD8 effector cell frequency (identified as $CD44^{high}CD62L^{low}$) was reduced in the PLN of NOR MSC-treated mice, while no difference was detected in Treg frequency ($n = 5$, $P = 0.041$ and $P = 0.0022$ for CD4 and CD8 effectors, respectively). *C*: CD11c single-positive cells and $CD11c^{high}CD11b^{+}$ cells were reduced in response to NOR MSC therapy ($n = 5$, $P = 0.004$ and $P = 0.015$, respectively) while the $CD11c^{low}CD11b^{+}$ population was increased ($n = 5$, 1.12 ± 0.1% and 2.06 ± 0.34% for control and NOR MSC-treated, respectively, $P = 0.029$). Analysis of the CD11c single-positive population showed a marked increase in the plasmacytoid DC subset in NOR MSC-treated mice (gated on $CD11c^{+}CD11b^{-}$, followed by analysis of B220 expression, $n = 5$, 21.9 ± 3.18% and 37.5 ± 3.31% for control and NOR MSC-treated, respectively, $P = 0.0094$). The frequencies of $CD11b^{+}Ly\text{-}6c^{+}$ monocytes (n = 3–5, $P = 0.042$) and $F4/80^{+}$ macrophages ($n = 5$, $P = 0.015$) were also found to be reduced in response to NOR MSCs. All data are displayed with means and SEM.

DISCUSSION

The incidence of type 1 diabetes is steadily rising at a global level (33–35), and the most common form of intervention seeking to reverse the disease in recently diagnosed patients has been that of immunosuppression through use of agents such as anti-CD3. However, as immunosuppressive regimens are commonly associated with acute morbidity, novel treatments to reduce the burden of immunosuppression are in dire need of development. MSC therapy is one such treatment modality that, because of the considerable immunomodulatory effects of these cells, has shown promising results in treating autoimmune diseases and has the potential to serve as a component of combination therapy to reduce immunosuppressive regimen morbidity (9–12). Whereas MSCs are capable of differentiating into a number of mesenchymal cell lineages, hematopoietic stem cells are multipotent stem cells that give rise to all cells in the blood and that have been shown to have immunomodulatory roles as well; indeed, hematopoietic stem cell transplantation in patients with newly diagnosed type 1 diabetes has resulted in improved β-cell function (36). Clinical trials examining the effects of MSC therapy have also been initiated for a multitude of disorders, including type 1 diabetes. However, type 1 diabetes trials have been initiated with a paucity of preclinical data, which are necessary to determine the type and course of MSC therapy as well as to elucidate the mechanisms by which MSCs exert their immunomodulatory effects. We and others have previously demonstrated the benefit of MSC therapy in the specific setting of type 1 diabetes (13,14,37,38). Importantly, our previous work demonstrated no therapeutic benefit of autologous MSCs in vivo using a NOD mouse model (13). Conversely, allogeneic BALB/c MSC treatment was efficient in the treatment of type 1 diabetes, but reversal was short-lived, perhaps due to the eventual rejection of the allogeneic cells. Our previous work also indicated that the most significant preventative effect on experimental type 1 diabetes occurred with congenic NOR MSC treatment. Given these data, we sought to examine the effect of NOR MSCs on reversal of hyperglycemia as well as to elucidate the mechanisms responsible for NOR MSC immunomodulation.

In this report, we first demonstrate that NOR MSCs are functionally and lineally mesenchymal as well as confirm their immunomodulatory function in suppressing nonspecific TCR-stimulated proliferation. We next examined their immunosuppressive ability in the specific context of autoreactive T-cell proliferation through use of the BDC2.5 autoreactive T-cell assay; NOR MSCs were shown to potently suppress diabetogenic T-cell proliferation and IFN-γ production. Although NOR MSCs expressed PD-L1

at low levels at baseline, treatment with recombinant IFN-γ resulted in abundant PD-L1 expression. Similarly, previous reports have demonstrated IFN-γ to be important for MSC-mediated immunosuppression or PD-L1-mediated MSC immunoregulation (6,39,40). In our model, IFN-γ production by autoreactive T-cells may upregulate PD-L1 expression on MSCs and thereby augment their immunomodulatory capability. Indeed, we show that addition of NOR MSCs to the BDC2.5 autoreactive assay results in prodigious upregulation of PD-L1 expression on NOR MSCs; we postulate that a similar upregulation occurs after in vivo administration of MSCs to diabetic mice. With regard to functionality, the immunosuppressive effect of NOR MSCs was shown to be mediated in part via PD-L1 through siRNA knockdown of MSC PD-L1 expression and consequent abrogation of the suppressive effect on autoreactive T-cell proliferation. Given these results, further exploration of the effects of administering PD-L1-positive MSCs and the consequent potential for reducing the MSC number injected to diabetic NOD mice is certainly of merit. We also investigated the role of MSCs in regulating the phenotype of DCs. Primary diabetic insult is identified by peri-insulitis of the pancreas after DC and macrophage infiltration, and MSCs have been shown to both suppress and alter DC differentiation in other models (2,7). Herein, we show that the presence of MSCs reduces CD11c and CD11b expression, decreases inflammatory Ly-6c expression in the $CD11b^+$ population, and suppresses differentiation as shown by increased frequency of lineage-negative and $Sca\text{-}1^+$ cells. TNF-α production, a growth factor involved in DC maturation and produced by mature DCs, was suppressed in MSC cocultures, and DC morphology was similarly altered in the presence of MSCs. Moreover, we demonstrate that these effects are in large part mediated by IL-6 through the use of a blocking IL-6 antibody and the consequent reversal of this effect, although other factors such as Flt3L and M-CSF may be involved due to incomplete abrogation of all phenotypic alterations.

Our reversal studies in the NOD mouse show a marked incidence in reversal of recent-onset hyperglycemia in eight of nine mice in response to NOR MSC treatment, with prolonged reversal of 83% of mice treated with NOR MSCs (i.e., in five of six long-term survivors), whereas control mice reverted to hyperglycemia soon after cessation of insulin replacement. Serum cytokine studies of reversal mice showed changes in the cytokine profile with respect to IL-6, IL-7, IL-10, and IL-12(p40). IL-6 is a pleiotropic cytokine purported to have both anti- and proinflammatory roles; in type 1 diabetes, reports of its effects are conflicting, but it has been shown to protect β-cells from apoptosis and impaired function as well as delay the onset of overt diabetes in NOD mice (41–43). Our in vitro data also demonstrate the central role of IL-6 in NOR MSC suppression of DC generation. IL-7, although important for effector memory T-cell survival, has also recently been demonstrated to be necessary for common lymphoid progenitor development, from which plasmacytoid DCs arise (44). IL-10 is an established immunoregulatory cytokine, whereas the homodimer IL-12(p40) inhibits action of the bioactive heterodimer IL-12(p70) (45,46). Taken together, changes in cytokines appear to skew the inflammatory diabetogenic environment toward a more regulatory profile in response to NOR MSC treatment. Consistent with our in vitro data, we demonstrate altered DC phenotype in response to NOR MSC treatment, manifested by diverting the $CD11c^+$ subset toward a more regulatory cell as well as possibly preventing myeloid APC differentiation, as demonstrated by reduced expression of CD11c and F4/80 after MSC injection in vivo. Interestingly, the $CD11c^+CD11b^+$ subset has been shown to prime autoreactive T-cells, resulting in physiological β-cell death (47), and our treatment, particularly given our in vitro data, appears to retard development of this cell subset. Moreover, plasmacytoid DC frequency was found to be markedly increased in response to NOR MSC treatment, and DCs of this lineage have been shown to prevent acceleration of insulitis in NOD mice, as well as to suppress myeloid DC activation of effector cells (48,49), which is consistent with the decreased frequency of CD4 and CD8 effectors that we observed in response to NOR MSC treatment. As it has also been shown that type 1 diabetic patients have a reduced pDC compartment (50), increased frequency of pDC after NOR MSC treatment may thus contribute to the reversal of hyperglycemia that we observed.

Taken together, NOR MSC treatment resulted in efficient reversal of hyperglycemia, suppressed autoreactive T-cell proliferation via PD-L1, and increased production of regulatory cytokines and frequency of plasmacytoid DCs. This work is the first to demonstrate the distinct benefit of congenic MSCs in reversing hyperglycemia and ameliorating diabetes pathogenesis. Further exploration to optimize and to confirm the safety and efficacy of MSC therapy is the subject of our future studies. These data should serve to shape future type 1 diabetes clinical trials with regard to optimal MSC source and therapeutic regimen.

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M.J. researched data, contributed to discussion, and wrote the manuscript. S.Y., R.M., and J.A. researched data. A.A. and J.G. researched data and contributed to discussion. P.F. contributed to discussion. M.A. reviewed and edited the manuscript. M.H.S. contributed to discussion and reviewed and edited the manuscript. R.A. contributed to discussion and wrote the manuscript.

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